

Award Number: DAMD17-01-1-0520

TITLE: Rapid Generation and Screening of Novel Bi-Specific  
Single-Chain FV Molecules Capable of Inhibiting the  
Growth of Breast Cancer

PRINCIPAL INVESTIGATOR: Gregory P. Adams, Ph.D.

CONTRACTING ORGANIZATION: Fox Chase Cancer Center  
Philadelphia, Pennsylvania 19111

REPORT DATE: May 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20030923 102

# REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE May 2003	3. REPORT TYPE AND DATES COVERED Final (1 May 2001 - 30 Apr 2003)	
4. TITLE AND SUBTITLE Rapid Generation and Screening of Novel Bi-Specific Single-Chain FV Molecules Capable of Inhibiting the Growth of Breast Cancer			5. FUNDING NUMBERS DAMD17-01-1-0520	
6. AUTHOR(S) Gregory P. Adams, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Fox Chase Cancer Center Philadelphia, Pennsylvania 19111  E-Mail: gp_adams@fccc.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words)  Antibodies that perturb signal transduction of cancer cells have demonstrated significant utility in the treatment of breast cancer and lymphoma. As signal transduction in the Epidermal Growth Factor Receptor (EGFR) family (EGFR, HER2, HER3, HER4) involves ligand binding and subsequent heterodimerization of two members, the most potent monoclonal antibody (MAb)-based agent would likely be one that also mediates a similar crosslinking event. <i>The fundamental hypothesis underlying this Concept Award Project was that signal transduction through components of the EGFR family could be manipulated through the construction of novel bispecific antibodies that engage multiple epitopes of this family.</i> The goals of this proposal were to develop a novel, rapid methodology to create bispecific single-chain Fv (bs-scFv) molecules using molecular shuffling of two large groups of scFv (libraries) specific for HER2 and HER3 and to perform preliminary evaluations of the in vitro specificity and anti-tumor effects against cells that over express both target antigens.				
14. SUBJECT TERMS Bispecific antibody, HER2, HER3				15. NUMBER OF PAGES 10
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

## Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	9
Reportable Outcomes.....	9
Conclusions.....	10
References.....	10
Appendices.....	

## Introduction

Antibodies that perturb signal transduction of cancer cells have demonstrated significant utility in the treatment of breast cancer and lymphoma. As signal transduction in the Epidermal Growth Factor Receptor (EGFR) family (EGFR, HER2, HER3, HER4) involves ligand binding and subsequent heterodimerization of two members, the most potent monoclonal antibody (MAb)-based agent would likely be one that also mediates a similar crosslinking event. *The fundamental hypothesis underlying this Concept Award Project was that signal transduction through components of the EGFR family could be manipulated through the construction of novel bispecific antibodies that engage multiple epitopes of this family.* The goals of this proposal were to develop a novel, rapid methodology to create bispecific single-chain Fv (bs-scFv) molecules using molecular shuffling of two large groups of scFv (libraries) specific for HER2 and HER3 and to perform preliminary evaluations of the in vitro specificity and anti-tumor effects against cells that over express both target antigens.

## Body

### Research Accomplishments:

*Task 1. Isolate scFv clones from a large human scFv library that are reactive with HER3.* We used human HER3 extracellular domain (ECD) as a target to pan a phage display library. A number of scFv were isolated that bind to HER3 (Table 1). PCR fingerprinting revealed that 6 contained unique sequences. Note: while it was not directly identified in the scope of the proposal, we also isolated scFv molecules that bound to HER4 ECD (10 unique clones) and EGFR ECD (33 unique clones).

Table 1. Properties of scFv sublibraries.

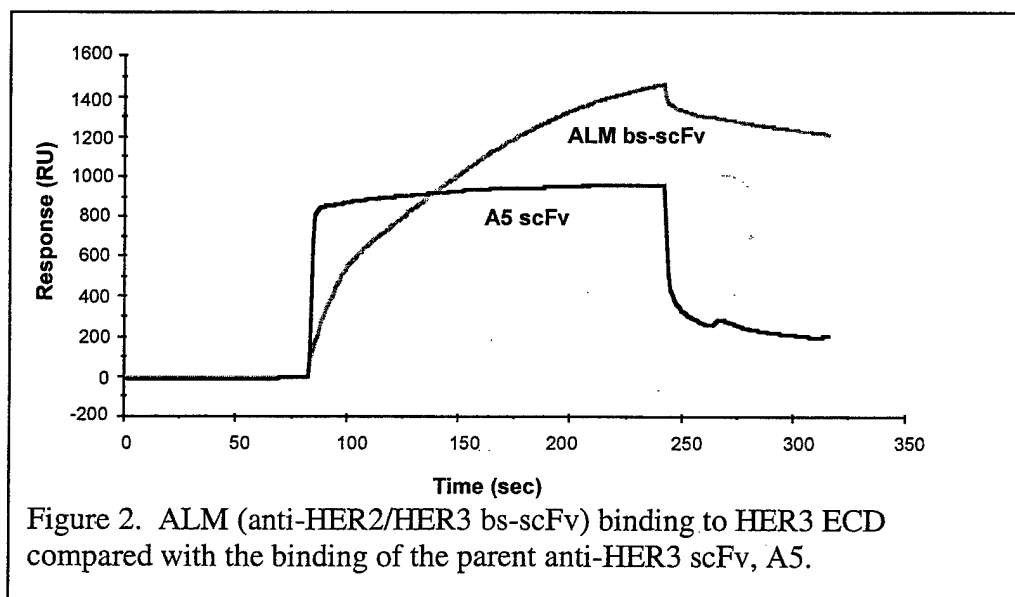
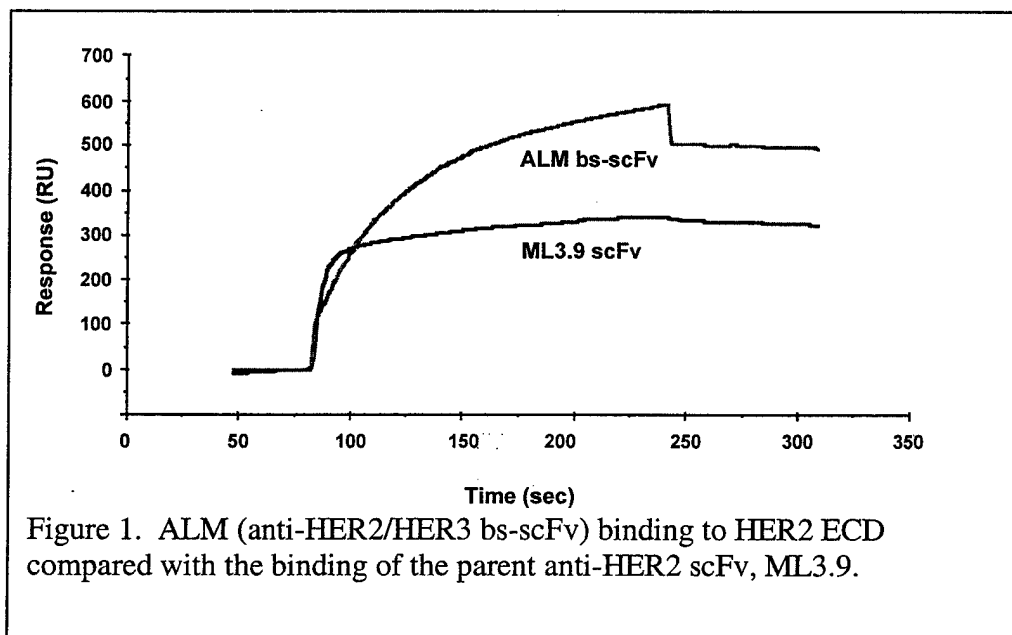
Antigen	# of scFv libraries screened	# Unique scFv clones in sublibrary
HER2	2	2*
HER3	1	6
HER4	1	10
EGFR	3	33

\*Note, 75 affinity mutants ranging in affinity from the mM to pM level have been created from one of these clones

*Task 2. Create bs-scFv molecules using scFv shuffling.* A unique peptide spacer with the amino acid sequence "NSGAGTSGSGASGEGSGSKL" was designed that would impart flexibility and resistance to serum proteases. Based upon incompatible restriction sites between the scFv clones, scFv shuffling techniques required extensive sight-directed mutagenesis on almost every clone to alter restriction sites. This effort proved to be counter productive to the goal of speeding up the partnering and screening processes. We have recently initiated a new method of crosslinking pairs of scFv using a monoclonal antibody directed against a tag of the carboxy terminus. It is our belief that this will accomplish the same goal as the scFv shuffling would have – to identify optimal pairs of scFv for bs-scFv production. In the meantime, we selected a few anti-HER3 and anti-HER4 scFv molecules and used these together with the peptide spacer (shown above) and our best anti-HER2 scFv (ML3.9) to create a candidate bs-scFv that were screened for in

vitro specificity and efficacy. As a result of this effort, we have now created two bs-scFv that are specific for HER2 and HER3 (ALM and FLM), two bs-scFv that are specific for HER2 and HER4 (BLM and ELM), two homodimeric scFv that are specific for HER3 (ALA and FLF), one homodimeric scFv that is specific for HER2 (MLM) and one heterodimeric scFv that is specific for two epitopes on HER3 (FLM).

Task 3. Evaluate the specificity of the bs-scFv molecules for both target antigens. The specificity of the first bs-scFv, ALM, for HER2 and HER3 ECDs was evaluated by surface plasmon resonance on the BIAcore instrument. It was found to be capable of binding to HER2 ECD, HER3 ECD and of forming a complex between HER2 and HER3 ECDs in a sandwich assay (Figures 1-3). The specificity of BLM for HER2 and HER4 was similarly evaluated (Figure 4).



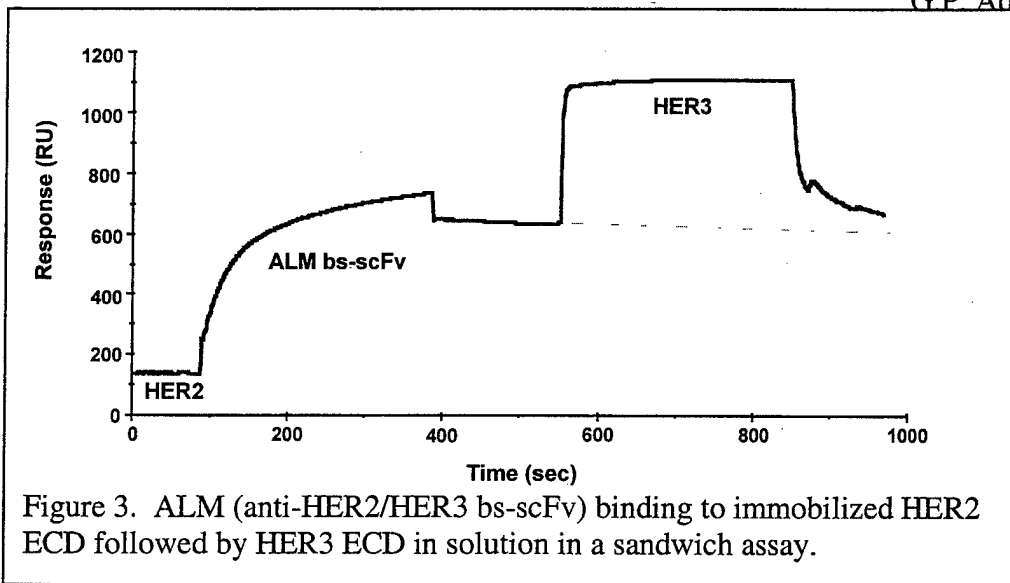
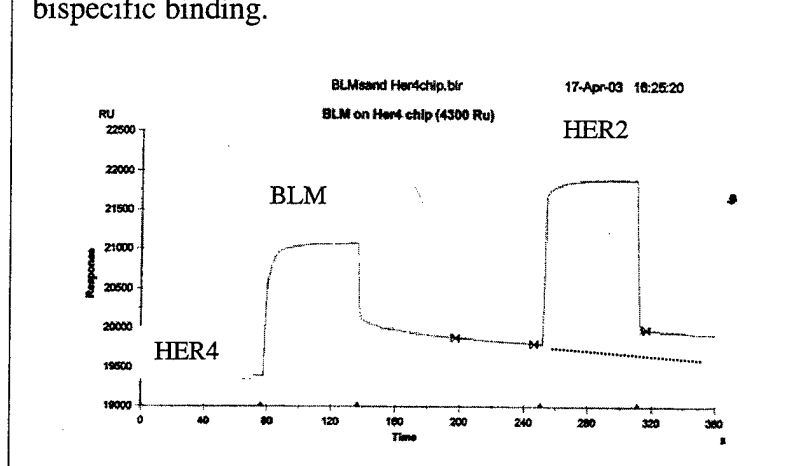


Figure 4. BIAcore sensogram trace of the BLM anti-HER2/HER4 bs-scFv molecule demonstrates bispecific binding. BLM binds to HER4 that is immobilized on the chip and then binds the subsequently applied HER2. An extrapolation of the BLM dissociation (dotted line) reveals bispecific binding.



**Task 4. Evaluate the function of the bs-scFvs.** The functional consequences of the binding of first bs-scFv, ALM, to cells that overexpress HER2 and HER3 was evaluated in MTT assays, a clonogenicity assay and by flow cytometry. In a five day MTT assay, which is a measure of impact on cellular proliferation, ALM mediated a similar degree of growth inhibition of human breast tumor BT-474 cells as did Herceptin – a clinically approved MAb that targets HER2 (Figure 5). Similar results were seen with the BLM (ant-HER2/HER4) bs-scFv (Figure 6). In a 17 day clonogenicity assay, ALM incubated with BT-474 breast cancer cells at a roughly equimolar concentration to the amount of cell-surface HER2 lead to approximately a 50% reduction in tumor cell survival (Figure

7). Increasing the quantity lead to a decrease in efficacy. Finally, the impact of ALM on HER2 and HER3 expression on BT-474 cells was determined by flow cytometry. It was found that 1.6 ug/ml ALM incubated with  $1.6 \times 10^6$  BT-474 cells resulted in a reduction of cell-surface HER2 after 4 hr @ 37°C and a reduction in cell surface HER3 after 48 hr @ 37°C (Figure 8).

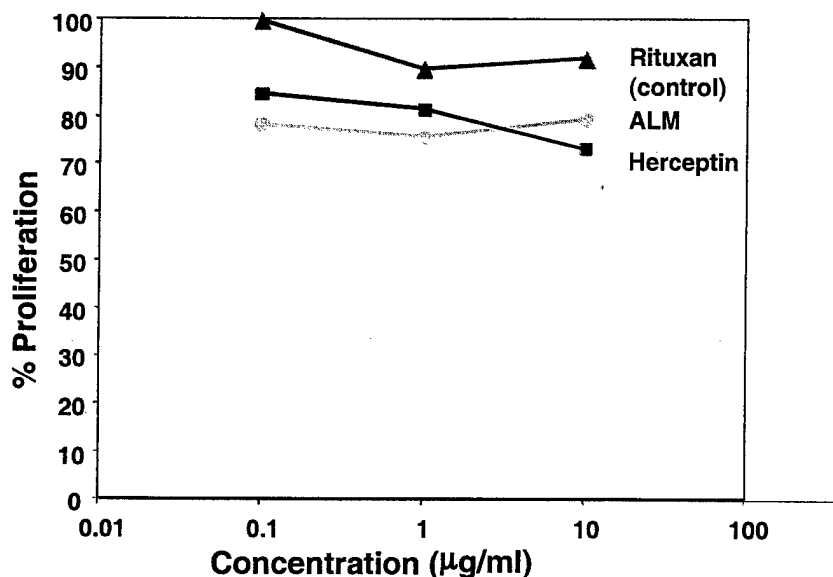
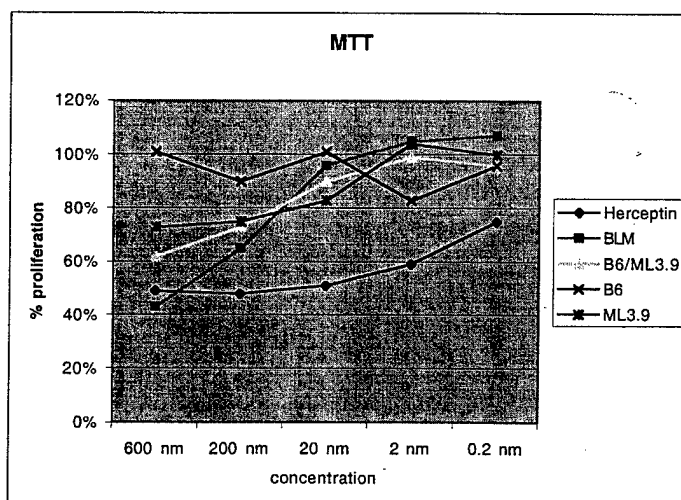


Figure 5. In an MTT assay, ALM has a similar anti-proliferative effect on human breast tumor BT-474 cells as does Herceptin. Rituxan is included as a negative control.

Figure 6. Representative cell proliferation (MTT) assay, using BT-474 breast cancer cell line, demonstrates that the growth inhibition properties of bs-scFv BLM (anti-HER4/anti-HER2) are superior to each scFv alone (B6 anti-HER4 or ML3.9 anti-HER2) or the combination of both scFv (B6/ML3.9) and are equal to Herceptin (positive control) at the highest dose.



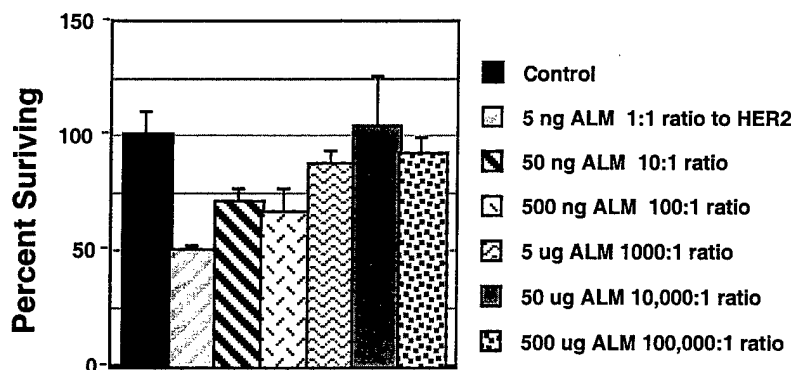


Figure 7. Results of a 17 day clonogenicity assay using increasing concentrations of ALM with BT-474 cells. Ratios of ALM to HER2/*neu* are indicated,  $n=3$  plates/concentration.

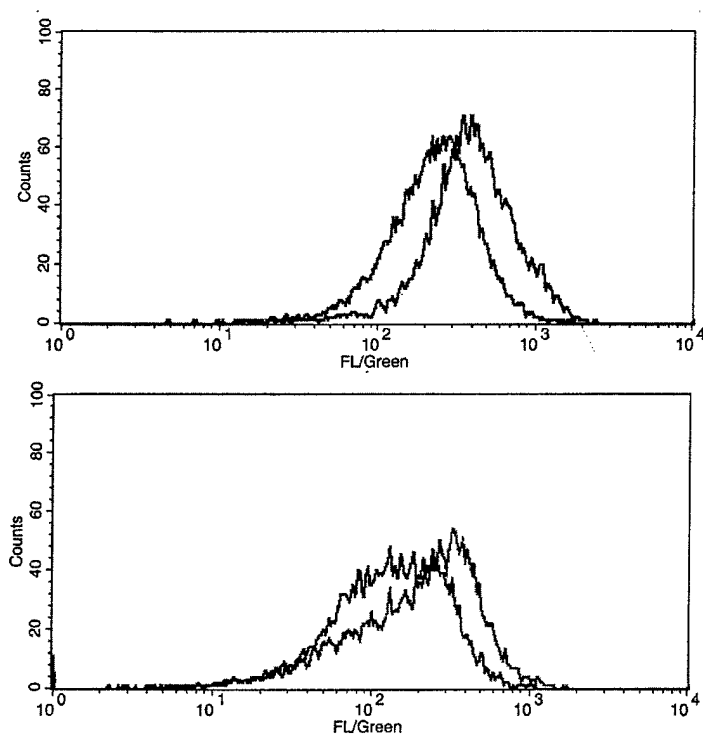


Figure 8. Impact of ALM on the expression of HER2 at 4 hrs (top) and HER3 at 48 hrs (bottom) on the surface of BT-474 cells. The left-hand peaks on each plot are with ALM, the right-hand peaks are without ALM. 1.6  $\mu\text{g}/\text{ml}$  ALM incubated with  $1.6 \times 10^6$  BT-474 cells, HER2 detected with 520C9 MAb and HER3 detected with AB4 (Neomarkers).



### **Key Research Accomplishments**

- Isolated scFv from a human phage display library that are specific for HER3, HER4 and EGFR. 6 unique clones that bind to HER3, 10 unique clones that bind to HER4 and 33 unique clones that bind to EGFR.
- Developed a novel peptide spacer that is expected to be protease resistant for use in linking the scFv into a bispecific format (Spacer sequence = NSGAGTSGS GASGEGSGSKL)
- Created the following scFv-based molecules; two bs-scFv that are specific for HER2 and HER3 (ALM and FLM), two that are specific for HER2 and HER4 (BLM and ELM), two homodimeric scFv that are specific for HER3 (ALA and FLF), one homodimeric scFv that is specific for HER2 (MLM) and one heterodimeric scFv that is specific for two epitopes on HER3 (FLM).
- Evaluated the impact of the lead bs-scFv molecule, ALM, on the in vitro growth properties of the human breast tumor cell line, BT-474, that overexpresses HER2 and HER3.
  - In an MTT assay evaluating the impact on cell proliferation, a five day incubation with ALM, Herceptin (positive control) or Rituxan (negative control) lead to comparable inhibition of proliferation with ALM and Herceptin.
  - In a clonogenicity assay, a 17 day incubation with 5 ng to 500 ug concentrations of ALM (ALM to cell surface HER2 ratios of 1:1 to 100,000:1) lead to approximately 50% reduction in cell viability in the group treated with the lowest concentration of ALM. Interestingly, as the concentration was increased, the impact on survival decreased.
  - Have determined by flow cytometry that incubation of BT-474 cells with ALM leads to decreases in the cell surface expression of HER2 and HER3.
- Evaluated the impact of a second bs-scFv molecule, BLM, on the in vitro growth properties of the human breast tumor cell line, BT-474, that overexpresses HER2 and HER4.
  - In an MTT assay evaluating the impact on cell proliferation, a five day incubation with ALM, Herceptin (positive control) or Rituxan (negative control) lead to comparable inhibition of proliferation with BLM and Herceptin.

### **Reportable Outcomes**

- American Association for Cancer Research abstract. #4810. Production and Evaluation of Bispecific single-chain Fv molecules that target HER2/neu and HER3. E. Horak, L. Shahied, C. Shaller, A. Tesfaye, H. Simmons, R. Alpaugh, N. Greer, T. Heitner, J. Garrison, J. Marks, L. Weiner and G. Adams. Proc. AACR. 43:971, 2002.
- Presentation at "AntibOZ", An International Forum: Predicting the Next Wave of Protein-Based Therapies and Immunodiagnostics. Heron Island, Queensland Australia. April 8-12<sup>th</sup>, 2002.

- Presentation at Keystone Meeting Feb, 2003 in Banff Canada. "Targeting the Epidermal Growth Factor Receptor Family with Heterodimeric Single-chain FV molecules. Eva M Horak, Matthew K Robinson, Tara Heitner, Jennifer L Garrison, Lillian S Shahied, Calvin C Shaller, Abohawariat Tesfaye, Heidi H Simmons, R Katherine Alpaugh, Nathaniel B Greer, James D Marks, Louis M Weiner and Gregory P Adams.
- Patent applied for April 5, 2002, Serial No. 60/370,276.

#### **List of Personnel**

Abohawariat Tesfaye, Postdoctoral Associate

#### **Conclusions**

The generation of a scFv shuffling system for creating multiple bs-scFv partners was more difficult than expected. While progress has been made in this area, we have proceeded to make two bs-scFv molecules that target HER2 and HER3, two that target HER2 and HER4 and homodimeric controls that target HER2 or HER3. The lead molecule, ALM, has been evaluated for specificity and efficacy in vitro. We have found that it binds specifically to both targets and mediates a decrease in cell surface expression of HER2 and HER3 and leads to a reduction in cell survival and proliferation. We plan to evaluate the in vivo efficacy of ALM in immunodeficient mice bearing s.c. human breast cancer tumors. If ALM shows a similar effect in vivo, we will plan on initiating clinical trials as soon as possible.

#### **References**

N/A